[19]中华人民共和国国家知识产权局

[51]Int.Cl[7]
G01N 33/68

[12]发明专利申请公开说明书

[21]申请号 00105820.7

[43]公开日 2000年11月22日

[11]公开号 CN 1274085A

[22]申请日 2000.4.13 [21]申请号 00105820.7

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[54]发明名称 一种蛋白芯片及其制备方法并用其筛选单克隆抗体

[57]摘要

本发明涉及一种蛋白芯片及其制备，并用其筛选单克隆抗体的灵敏方法，包括步骤：

①用组织匀浆免疫BALB/c小鼠；

②取BALB/c小鼠的脾细胞和骨髓瘤细胞融合；

③用流式细胞仪分离单个杂交瘤细胞，扩增培养；

④制备蛋白芯片，用来鉴定产生的抗体，同时建立杂交瘤细胞库；

⑤制备筛选用蛋白芯片，用特异性抗原和多克隆抗体筛选对应的单克隆抗体。

本发明用于单克隆抗体的大规模快速筛选。

ISSN1008-4274

知识产权出版社出版

00-04-13

权 利 要 求 书

1、一种蛋白芯片，它是将大量特定的抗原或抗体，有序地固化在载体上，载体可以为硝酸纤维素膜、尼龙膜或玻片，利用抗原抗体的亲和反应检测对应的抗体或抗原的阵列，其特征是利用这种蛋白芯片可以大规模筛选单克隆抗体，是一种快速灵敏的筛选方法，具体分为五个步骤：

①用组织匀浆免疫BALB/c小鼠；

②取BALB/c小鼠的脾细胞和骨髓瘤细胞融合；

③用流式细胞仪分离单个杂交瘤细胞，扩增培养；

④制备蛋白芯片，用来鉴定产生的抗体，同时建立杂交瘤细胞库；

⑤制备筛选用蛋白芯片，用特异性抗原和多克隆抗体筛选对应的单克隆抗体；这样的筛选流程。

2、根据权利要求 1 所述的这种蛋白芯片的制备方法：其特征是将免疫过的BALB/c小鼠的脾细胞和骨髓瘤细胞融合产生的杂交瘤细胞，分离出的单个细胞，培养在384孔板中，

取培养杂交瘤细胞的上清液转移到新的384孔板，用点样器把上清液点在玻片上形成1000点/cm^2的阵列，每个点对应一个孔，每点为0.2-1μl上清液，IgG的浓度为1-100Pg/ml，一次可以制备多张蛋白芯片。

3、根据权利要求 1 所述的用这种蛋白芯片鉴定产生的抗体的方法，其特征是用蛋白芯片加FITC即异硫氰酸荧光素标记的兔抗鼠IgG抗体，效价为1：50-1：1000，37℃湿盒孵育30-60分钟，在荧光显微镜下观察结果。

4、根据权利要求 1 所述的用这种蛋白芯片鉴定产生的抗体的方法，其特征是在鉴定产生的抗体的同时，还建立了杂交瘤细胞库，具体方法为：在荧光显微镜下挑选有荧光的点，标记对应的杂交瘤细胞孔，取适量细胞，扩增培养，液氮冻存细胞，建立杂交瘤细胞库。

5、根据权利要求 1 所述的用这种蛋白芯片筛选单克隆抗体的方法，其特征是用特异性抗原和多克隆抗体筛选对应的单克隆抗体，具体方法为：

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1）在蛋白芯片上加抗原，浓度为0.1-100Pg/ml，37℃湿盒孵育30-60分钟；

2）用磷酸盐缓冲液洗芯片3-5次；

3）在蛋白芯片上加兔抗特异性抗原的多克隆抗体，抗体用FITC标记，抗体效价为1：5-1：1000，37℃湿盒孵育30-60分钟；

4）荧光显微镜下观察蛋白芯片，挑选荧光显色的斑点，从杂交瘤细胞库中取出对应的杂交瘤细胞，扩增培养，鉴定筛选出的单克隆抗体；

5）换另一种抗原和其荧光标记的多克隆抗体，重复1）－4）步骤，筛选出另一种单克隆抗体。

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说 明 书

一种蛋白芯片及其制备方法并用其筛选单克隆抗体

本发明属分子生物学领域，涉及一种蛋白芯片及其制备方法，并用其来大规模快速地筛选单克隆抗体的灵敏的方法。

单克隆抗体技术发明于1975年，其制备过程基本如下：

①用抗原免疫小鼠，②取小鼠的脾淋巴细胞和骨髓瘤细胞融合，③杂交瘤细胞克隆化，④筛选特异的单克隆抗体。

克隆化方法有：①有限稀释法，②软琼脂培养法，③单细胞显微操作法，④单克隆细胞集团显微操作法，⑤萤光激活细胞分类仪（流式细胞仪）分离法。

经典的筛选特异性单克隆抗体的方法有：①免疫酶技术、②免疫荧光技术、③放射免疫测定、④化学发光免疫分析、⑤间接凝血试验。其中免疫酶技术用得最多，现以免疫酶技术的一种技术——间接酶联免疫分析（ELISA）来说明筛选过程。用抗原包被载体，加入待测的单克隆化杂交瘤细胞的培养上清液，若上清液中有对应的单克隆抗体，则与包被的抗原结合；洗涤后再加入酶标记的第二抗体，形成固定化的抗原－抗体－酶标记的第二抗体复合物；依据底物显色反应的强度，可对待测的单克隆抗体样品进行定性或定量分析。

经典的筛选特异性单克隆抗体的方法最大的缺点是得到的数个克隆中只能筛选出一种抗原的单克隆抗体、浪费大、效率低。

应用蛋白芯片能快速检测大量抗原抗体的结合反应。如果用组织匀浆免疫小鼠，把克隆化的杂交瘤细胞分泌的抗体固化到载体（通常为尼龙膜、硝酸纤维膜或玻片）上，用不同抗原和其多克隆抗体便可筛选到不同抗原的单克隆抗体的杂交瘤细胞株。从理论上讲，如果克隆化的杂交瘤细胞库足够大，可筛选到所有蛋白质的单克隆抗体。

经检索提供的对比文献及参考目录附后。

本发明的目的是发明一种蛋白芯片，通过其制备方法产生的蛋白芯片，能用于大规模快速地筛选单克隆抗体，方法简单，成本低，并

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且同时建立杂交瘤细胞库；本发明还涉及建立用特异性抗原和多克隆抗体筛选对应的单克隆抗体的方法。

为了实现本发明的目的采取的方案：

①用组织匀浆免疫BALB/c小鼠；

②取BALB/c小鼠的脾细胞和骨髓瘤细胞融合；

③用流式细胞仪分离单个杂交瘤细胞，扩增培养；

④制备蛋白芯片，用来鉴定产生的抗体，同时建立杂交瘤细胞库；

⑤制备筛选用蛋白芯片，用特异性抗原和多克隆抗体筛选对应的单克隆抗体。

结合附图对本发明进一步具体的说明：

所谓蛋白芯片即将大量特定的抗原（抗体），有序地固化在载体上，载体可以是硝酸纤维素膜、尼龙膜或玻片等其它载体，利用抗原抗体的亲和反应检测对应的抗体（抗原）的阵列。

本发明的流程和经典单克隆抗体制备的流程比较见图1。图中1为经典的杂交瘤抗体制备的流程图，2为本发明流程图，图中本发明①－⑤为五个操作步骤为：

①用组织匀浆免疫BALB/c小鼠；

②取BALB/c小鼠的脾细胞和骨髓瘤细胞融合；

③用流式细胞仪分离单个杂交瘤细胞，扩增培养；

④制备蛋白芯片，用来鉴定产生的抗体，同时建立杂交瘤细胞库；

⑤制备筛选用蛋白芯片，用特异性抗原和多克隆抗体筛选对应的单克隆抗体。

图1中：

3、组织匀浆　4、特种抗原　5、脾细胞　6、　杂交瘤细胞

7、克隆　8、单克隆抗体　9、流式分离仪分离单个杂交瘤细胞

10、蛋白芯片鉴定产生的抗体，建立杂交瘤细胞库

11、蛋白芯片筛选特异性单克隆抗体　12、融合

13、骨髓瘤细胞。

经典的制备方法也对应五个步骤，如图1中以上所注。

用蛋白芯片鉴定产生的抗体的原理见图2。

图 2 中：

1、为蛋白芯片筛选特异性单克隆抗体的示意图：

黑色圆点表示对应的孔有抗体产生；白色圆点表示无抗体产生，为哑克隆。

2、为显示无抗体产生时不显色的原理，当克隆中无小鼠的抗体IgG（免疫球蛋白）时，FITC（异硫氰酸荧光素）标记的抗小鼠IgG抗体不能结合，在洗涤时洗去，对应点无荧光显示。

3、为显示有抗体产生显色的原理，当克隆中有小鼠的IgG抗体时，把杂交瘤细胞培养的上清液顺序点印在玻片上，点印密度为1000点/cm^2，IgG会被固化在玻片上特定的位置；用FITC标记的兔抗鼠IgG在玻片上孵育，有鼠IgG的点便会有荧光，通过此方法可方便去掉大量的哑克隆。

4 为有效抗体，5 为无效抗体，6 为FITC标记的小鼠IgG抗体。

蛋白芯片筛选特异的抗体以AFP（甲胎球蛋白）为例，原理见图3，把产生IgG抗体的克隆上清液点印在玻片上，点印密度为1000点/cm^2，用AFP及标记FITC的免疫AFP多克隆抗体，在玻片上孵育，如果玻片上某点有AFP的单克隆抗体存在便会在激发光下产生荧光。

图 3 中，1 为蛋白芯片筛选特异性单克隆抗体的结果示意图，黑色表示阳性克隆，白色为阴性；2 为无抗体不产生显色原理图，非特异性单克隆抗体不能识别特异性抗原，故不能形成特异性单克隆抗体－抗原－多克隆抗体－荧光复合物，故对应点无荧光；3 为有抗体产生荧光显色原理图，特异性单克隆抗体能识别特异性抗原，故能形成特异性单克隆抗体－抗原－多克隆抗体－荧光复合物，故对应点有荧光；4 为特异性抗体，5 为非特异性抗体，6 为FITC标记的特异性抗原的抗体。

具体方法可细分为五部分：

1、用组织匀浆免疫BALB/c小鼠；

2、取BALB/c小鼠的脾细胞和骨髓瘤细胞融合；

3、用流式细胞仪分离单个杂交瘤细胞，扩增培养；

4、制备蛋白芯片，用来鉴定产生的抗体，同时建立杂交瘤细胞库；

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5、制备筛选用蛋白芯片，用特异性抗原和多克隆抗体筛选对应的单克隆抗体。

1、用组织匀浆免疫BALB/c小鼠：

取2克组织（各种正常组织及肿瘤组织均可）制成匀浆，离心，去掉大的细胞碎片，按常规免疫。

2、取BALB/c小鼠的脾细胞和骨髓瘤细胞融合：

取免疫BALB/c小鼠的脾细胞和适量的骨髓瘤细胞，用PEG（聚乙二醇）融合，融合后立即将细胞移入HAT培养液［含次黄嘌呤（H）、氨基喋呤（A）和胸腺嘧啶核苷（T）］中，4-5天换HT培养液，6-7天后换普通培养液。

3、用流式细胞仪分离单个杂交瘤细胞，扩增培养；

收集融合杂交瘤细胞，并培养7-8天，制备成悬液；用流式细胞仪分离单个细胞，收集在384孔培养板中，培养孔中需预先加入培养液和饲养细胞（通常用小鼠腹腔巨噬细胞）；培养5天，检测是否有抗体产生，培养8天再检测一次。

4、制备蛋白芯片，用来鉴定产生的抗体，同时建立杂交瘤细胞库；

①制备蛋白芯片：取培养细胞的上清液，转移到新的384孔板，用点样器把培养的上清液点在玻片上形成1000点/cm^2的阵列；（用不同的点样器，点印的密度和上清液的体积不同）；每个点对应一个孔，每个点为0.2-1μl的上清液。IgG的浓度为1-100Pg/ml。

②用制备的蛋白芯片鉴定产生的抗体，同时建立杂交瘤的细胞库：在蛋白芯片上加FITC标记的兔抗鼠IgG抗体（效价为1：50-1：1000），37℃湿盒孵育30-60分钟，用荧光显微镜观察结果（也可用其它方法获得图像，如激光扫描仪），

③建立杂交瘤细胞库：在荧光显微镜下，挑选有荧光的点；标记对应的杂交瘤细胞孔，取适量细胞，扩增培养，液氮冻存细胞，建立杂交瘤细胞库。

蛋白芯片鉴定产生抗体的荧光显微图象见图4，明亮斑点对应的杂交瘤产生抗体，否则为哑克隆。

用FITC标记的兔抗鼠IgG只是一种鉴定方法，其它方法包括用荧光分

子标记的其它抗小鼠IgG多克隆抗体，或者抗小鼠IgG多克隆抗体不标记，再偶联荧光标记的第二抗体；另一种方法为先加入抗小鼠IgG多克隆抗体，偶联生物素标记的第二抗体，再用荧光分子标记的亲和素显示荧光，以提高灵敏度。

5、制备筛选用芯片，用特异性抗原和多克隆抗体筛选对应的单克隆抗体。

①制备蛋白芯片：用384孔板复苏冻存细胞，培养5-10天，收集上清液，用点样器把培养细胞的上清液点在玻片上形成1000点/cm^2的阵列，每个点对应一个孔，每点为0.2-1μl上清液，IgG的浓度为1-100Pg/ml，一次可制备多张蛋白芯片。

②筛选特异性单克隆抗体：

1）在蛋白芯片上加抗原，浓度为0.1-100Pg/ml，37℃湿盒中孵育30-60分钟；

2）用磷酸盐缓冲液（PBS）洗芯片3-5次；

3）在蛋白芯片上加兔抗特异性多克隆抗体，抗体用FITC标记，抗体效价为1:5-1:1000，37℃湿盒孵育30-60分钟；

4）荧光显微镜下观察蛋白芯片，挑选荧光显色的斑点，从杂交瘤细胞库中取出对应的杂交瘤细胞，扩增培养，并鉴定，筛选出单克隆抗体；

5）换另一种抗原和其荧光标记的多克隆抗体，重复1）－4）步骤，可筛选另一种单克隆抗体。

实施例：

用胎肝匀浆免疫制备甲胎球蛋白（AFP）和肌动蛋白(Actin)的单克隆抗体。

实施例分五部分：

1、用胎肝匀浆液免疫BALB/c小鼠；

2、用BALB/c小鼠脾细胞和骨髓瘤细胞融合；

3、用流式细胞仪分离单个杂交瘤细胞，扩增培养；

4、制备蛋白芯片，去掉哑克隆，建立杂交瘤细胞库；

5、制备筛选用蛋白芯片，用AFP筛选AFP的单克隆抗体，用Actin

筛选Acin的单克隆抗体。

1、用胎肝匀浆液免疫BALB/c小鼠；

取2g流产胎儿胎肝组织，加10ml磷酸盐缓冲液研磨，超声破碎1-5分钟，离心去掉沉淀，用1升磷酸盐缓冲液透析3次，匀浆浓缩到1ml，免疫小鼠。免疫BALB/c小鼠5只，间隔2-4周再一次免疫，4周后静脉加强免疫。

2、取BALB/c小鼠脾细胞和骨髓细胞融合：

①制备小鼠腹腔巨噬细胞作为饲养细胞；

②5只BALB/c免疫小鼠，摘眼球放血，同时将小鼠处死；

无菌操作取脾，用网孔挤压法得到分散的脾细胞；

③融合前36-48小时，将骨髓瘤细胞扩大培养；

④分别取含1×10^8个脾细胞和$2-3\times10^7$个骨髓瘤细胞，加PEG融合，用HAT培养液筛选培养。

3、用流式细胞仪分离单个杂交瘤细胞，扩增培养；

①将细胞悬液加入已铺有饲养细胞层的７０ｃｍ培养皿，置于３７℃，含５－８％ CO_2的培养箱培养；

②融合当天HAT培养液培养，每天观察一次，第四天出现融合细胞的小集落克隆，立即换HT培养液，一天后再换普通完全培养液；

③制备小鼠腹腔巨噬细胞作饲养细胞，384孔板中每孔10^5个饲养细胞；

④在融合后的第六天，轻轻吹吸杂交瘤细胞，制成悬液；

⑤悬液用流式细胞仪分离成单个细胞，每孔中加入一个细胞，共100个384孔板；

⑥常规培养5-8天，观察克隆生长情况，第8天取培养上清液，转移到新的384孔板。

4、制备蛋白芯片，去掉哑克隆，建立杂交瘤细胞库：

①制备蛋白芯片：用点样器把培养上清液点印到玻片（美国Telechem公司，货号SMA-25）上，每个点0.2μl形成1000点/cm^2的阵列，每张玻片点印1920点（对应5块384孔板），共点印20张玻片，室温干燥24小时，加100mg/ml的牛血清蛋白，室温2小时封闭玻片，双蒸水缓慢冲洗2分钟；

②鉴定产生的抗体：芯片加上FITC标记的兔抗鼠IgG 0.3ml，效价为1:500，37℃湿盒孵育45分钟，磷酸盐缓冲液冲洗3次。

③荧光显微镜观察：用波长为492nm的光激发，加载525nm滤光片观察结果并照相，结果见图4，明亮斑点对应的杂交瘤有抗体产生，否则为哑克隆；

④建立杂交瘤细胞库：挑选表达IgG的孔（共6500孔），悬浮细胞，转到新的384孔培养板，共17块板，培养8天，转移上清液到新的384孔板，供制备蛋白芯片用，杂交瘤细胞再扩增培养，编号后冻存。

5、制备筛选用蛋白芯片，用AFP筛选AFP的单克隆抗体；用Actin筛选Actin的单克隆抗体：

①制备筛选用蛋白芯片：用点样器把上述获得的培养上清液点印到玻片上，每张玻片1920点，共4张玻片，重复点印1000套，室温干燥24小时，100mg/ml小牛血清封闭2小时，双蒸水缓慢冲洗2分钟，干燥保存。

②筛选AFP抗体

1）在芯片上加AFP抗原0.3ml浓度为20Pg/ml，37℃湿盒中孵育45分钟，PBS洗芯片三次；

2）用FITC标记兔抗AFP多克隆抗体（美国Chemicon公司，货号AB562），稀释到效价为1:100；

3）在芯片上加FITC标记的兔抗AFP多克隆抗体0.3ml，37℃湿盒孵育45分钟；

4）荧光显微镜观察芯片：激发光用492nm，加载525nm滤光片观察，并照相，记录显微荧光的位置，结果见图5，筛选到2株AFP单克隆抗体，分别用1.2表示。

③筛选Actin单克隆抗体：

程序同②，兔抗Actin多克隆抗体购自美国Chemicon公司，货号为AB978，结果见图6，筛选到4株Actin单克隆抗体，分别用1，2，3，4表示，从图中得1，2的亲和力最高，4的亲和力最低。

本发明的优点：

成本低廉：一次建库，用蛋白芯片可筛选到多种抗原的单克隆抗体，总体成本可成倍下降；

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操作简单：不重复免疫小鼠，不重复融合，建库后可多次筛选；

自动化：用流式细胞仪分离单个杂交瘤细胞；用蛋白芯片鉴定抗体，最大限度减少人力；能大规模快速地筛选单克隆抗体。

本发明的应用：

用本发明的蛋白芯片能大规模快速地筛选单克隆抗体，同时建立了杂交瘤细胞库。

说明书附图说明：

图 1 本发明的流程图和经典单克隆抗体制备流程图的比较：

1 为经典的杂交瘤抗体制备流程；2 为本发明的流程。①－⑤对应五个操作步骤。

①用组织匀浆免疫BALB/c小鼠；

②取BALB/c小鼠的脾细胞和骨髓瘤细胞融合；

③用流式细胞仪分离单个杂交瘤细胞，扩增培养；

④制备蛋白芯片，用来鉴定产生的抗体，同时建立杂交瘤细胞库；

⑤制备筛选用蛋白芯片，用特异性抗原和多克隆抗体筛选对应的单克隆抗体。

图 1 中：

3、组织匀浆　4、特种抗原　5、脾细胞　6、　杂交瘤细胞

7、克隆　8、单克隆抗体　9、流式分离仪分离单个杂交瘤细胞

1 0、蛋白芯片鉴定产生的抗体，建立杂交流细胞库

1 1、蛋白芯片筛选特异性单克隆抗体　1 2、融合

1 3、骨髓瘤细胞。

经典的制备方法也对应五个步骤，如图 1 中以上所注。

图 2 蛋白芯片鉴定产生抗体的原理图

1 为蛋白芯片检测结果示意图，黑色表示对应的孔有抗体产生，白色表示无抗体产生，为哑克隆。

2、显示无抗体产生时不显色的原理。当克隆中无小鼠的抗体IgG时，FITC标记的抗小鼠IgG抗体不能与其结合，对应的点无荧光显示。

3、显示有抗体产生时显色的原理，当克隆中有小鼠的IgG抗体时，FITC标记的抗小鼠IgG抗体与其结合，对应点有荧光显示。

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4、为有效抗体

5、为无效抗体

6、为FITC标记的兔抗鼠IgG抗体。

图 3 为蛋白芯片筛选特异性单克隆抗体的原理图。

1、为蛋白芯片筛选特异性单克隆抗体的结果示意图，黑色表示阳性克隆，白色表示阴性克隆。

2、为无抗体不产生显色原理图，非特异性单克隆抗体不能识别特异性抗原，故不能形成特异性单克隆抗体－抗原－多克隆抗体－荧光复合物，故对应点无荧光；

3、为有抗体产生荧光显色原理图，特异性单克隆抗体能识别特异性抗原，故能形成特异性单克隆抗体－抗原－多克隆抗体－荧光复合物，故对应点有荧光。

4、为特异性抗体

5、为非特异性抗体

6、为FITC标记的特异性抗原的抗体

图 4 为蛋白芯片鉴定产生的抗体的荧光显微镜图象。

明亮斑点对应的杂交瘤株有抗体产生，否则为哑克隆。

图 5 为蛋白芯片筛选AFP单克隆抗体。

筛选到 2 株AFP单克隆抗体，分别用1，2表示。

图 6 为蛋白芯片筛选Actin单克隆抗体

筛选到4株Actin单克隆抗体，分别用1，2，3，4表示，从图知1，2的亲和力最高而4亲和力最低。

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西安：陕西科学技术出版社，1992：42-73

4：对比文献：徐志凯等主编：实用单克隆抗体技术；

西安：陕西科学技术出版社，1992：65-73

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说 明 书 附 图

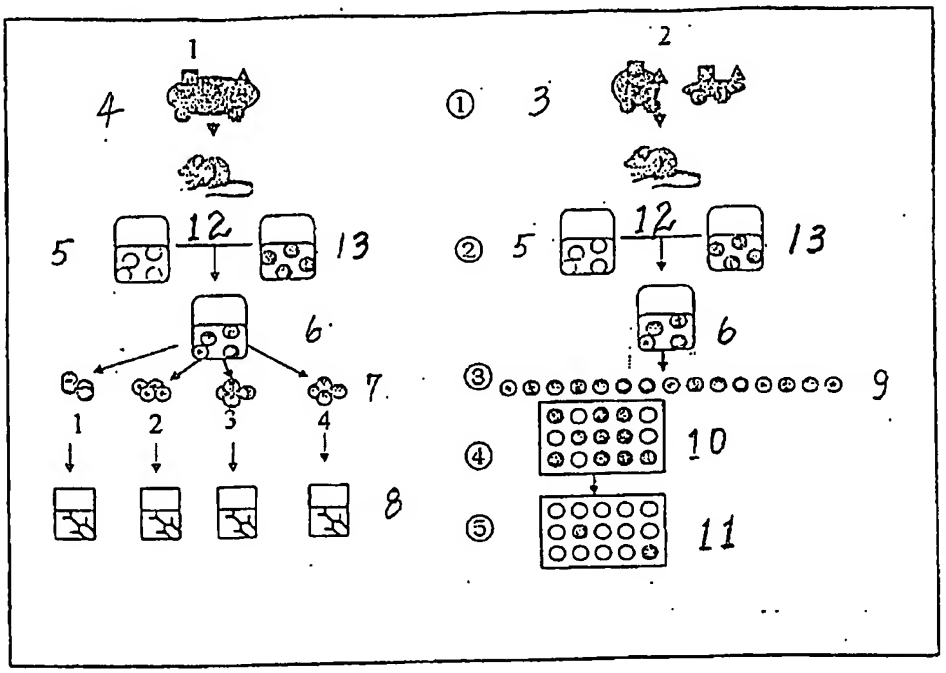

图1.

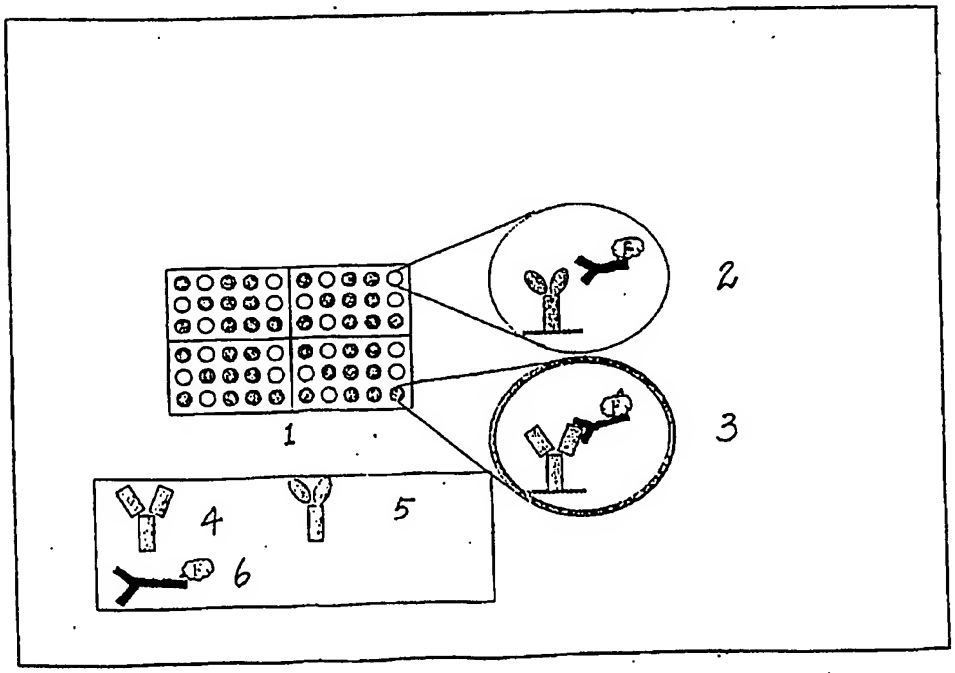

图2.

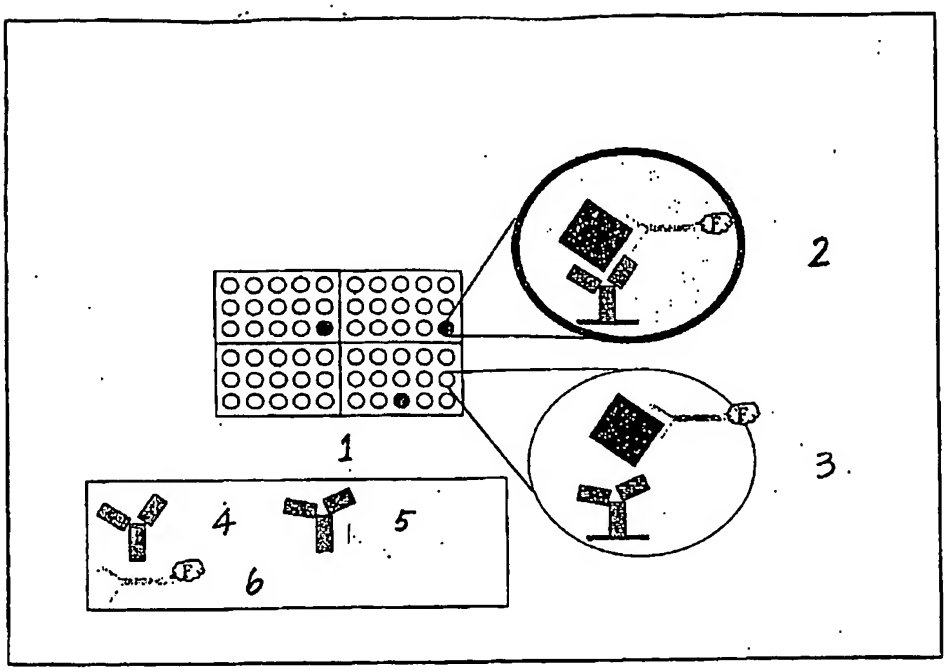

图3.

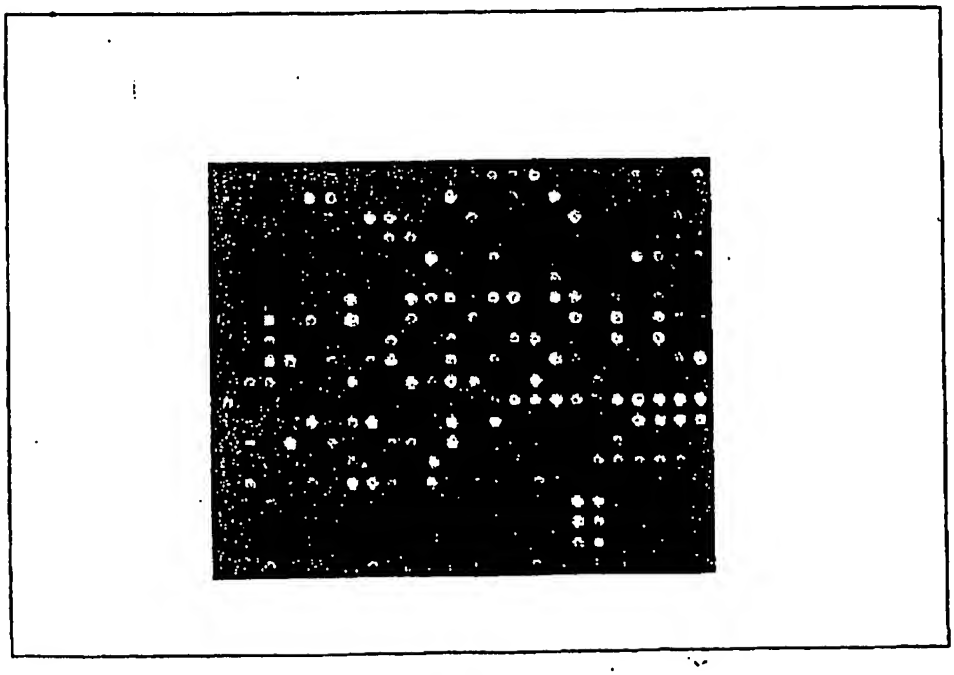

图4.

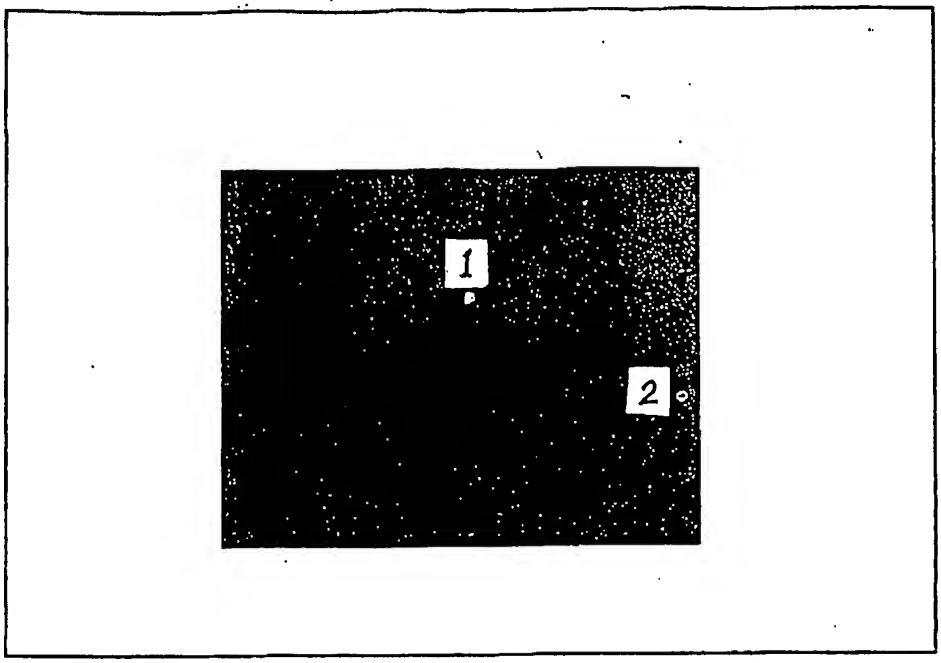

图5.

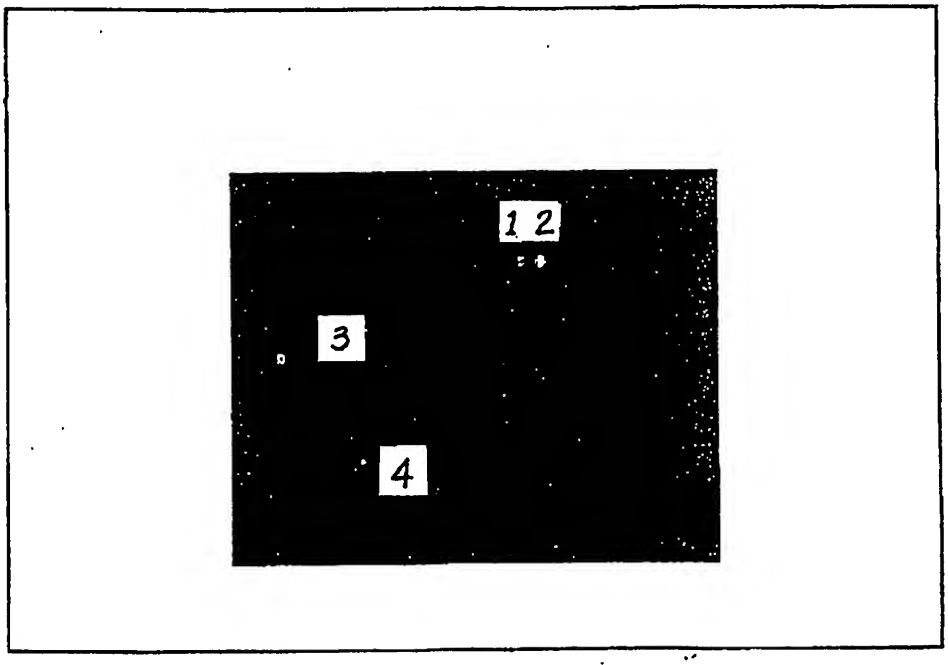

图6.

[19] National Intellectual Property Rights Bureau of the People's Republic of China

[51] Int. Cl[7]

G01N 33/68

[12] PUBLIC DESCRIPTION OF INVENTION PATENT APPLICATION

[21] Application No. 00105820.7

[43] Date of Publication: 22 November 2000 [11] Publication No. CN 1274085A

[22] Date of Application: 13.4.2000 [21] Application No.: 00105820.7 [71] Applicant: Chen Xueyin Address: Tumour Molecular Biology Laboratory, 27 Taiping Road, Beijing 100850 [72] Inventor: Chen Gaoming	[74] Patent agency: Sangao Patent Office Agent: Yao Baoqin Claims: 2 pages, Description: 9 pages, Appended Figures 3 pages

[54] Title of Invention: Protein chip and method for its preparation together with its use for screening monoclonal antibodies

[57] Abstract

This invention concerns a protein chip and method for its preparation together with its use in a sensitive method for screening monoclonal antibodies, including the steps:

(1) Immunizing BALB/c mice with homogenized tissue;

(2) Fusing spleen cells and myelomas of BALB/c mice;

(3) Separating individual hybridomas with a flow-type cell meter and raising a culture;

(4) Preparing a protein chip used for identifying the antibodies produced while at the same time establishing a hybridoma bank;

(5) Preparing a protein chip for screening use and using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies.

This invention is used for the large scale and rapid screening of monoclonal antibodies.

ISSN 1008-4274

Published by the Intellectual Property Rights Press

Claims

1. Protein chip, wherein a large quantity of specific antigens or antibodies are fixed in sequence on a carrier, which can be a cellulose nitrate film, a nylon film or a glass plate, to form an array utilizing the antigen-antibody affinity reaction to detect the corresponding antibody or antigen, characterized by utilizing this protein chip for the large scale screening of monoclonal antibodies, constituting a rapid and sensitive screening method, which in practice is divided into the following five steps:

(1) Immunizing BALB/c mice with homogenized tissue;
(2) Fusing spleen cells and myelomas of BALB/c mice;
(3) Separating individual hybridomas with a flow-type cell meter and raising a culture;
(4) Preparing a protein chip used for identifying the antibodies produced while at the same time establishing a hybridoma bank;
(5) Preparing a protein chip for screening use and using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies; thus constituting the screening process.

2. Preparation method for the protein chip according to Claim 1, characterized by fusing the spleen cells and myelomas of immunized BALB/c mice to produce hybridomas and separating out individual cells and culturing them in a 384 well plate,
Taking the supernatant fluid from the cultured hybridomas and transferring it to a new 384 well plate, and placing dots of the supernatant fluid on a glass plate to form an array of 1000 dots/cm^2 with a specimen dropper, such that each dot corresponds to a well, each dot being 0.2 - 1 μl of supernatant fluid with an IgG concentration of 1 - 100 Pg/ml, so that many protein chips can be prepared at one time.

3. Using the protein chip according to Claim 1 in a method for identifying antibodies produced, characterized by adding immune anti-mouse IgG antibodies labelled with FITC, namely fluorescein isothiocyanate, having a titre of 1 : 50 - 1 : 1000 to the protein chip, incubating for 30 - 60 minutes in an incubator at 37°C and observing the results under the fluorescence microscope.

4. Using the protein chip according to Claim 1 in a method for identifying antibodies produced, characterized by also establishing a hybridoma bank at the same time as identifying the antibodies produced, the actual method being: selecting fluorescing dots under the fluorescence microscope, labelling the corresponding wells containing the hybridomas, taking an appropriate quantity of cells and culturing them, and freezing the cells in liquid nitrogen to establish a hybridoma bank.

5. Using the protein chip according to Claim 1 in a method for screening monoclonal antibodies, characterized by using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies, the actual method being:

(1) Adding antigen to the protein chip at a concentration of 0.1 - 100 Pg/ml and incubating for 30 - 60 minutes in an incubator at 37°C;
(2) Washing the protein chip 3 - 5 times in a phosphate buffer solution;
(3) Adding polyclonal antibodies giving immunity to specific antigens to the protein chip, using FITC labelling for the antibodies, giving an antibody titre of 1 : 5 - 1 : 1000 and incubating for 30 - 60 minutes in an incubator at 37°C;
(4) Observing the protein chip under the fluorescence microscope, selecting fluorescing coloured dots and extracting the corresponding hybridomas from the hybridoma bank, culturing, and identifying the screened monoclonal antibodies;
(5) Substituting another antigen and its fluorescent labelled polyclonal antibody and repeating steps (1) - (4) to screen out another monoclonal antibody.

Description

Protein Chip and Method for its Preparation Together With its Use for Screening Monoclonal Antibodies

This invention falls within the area of molecular biology and concerns a protein chip and the method for its preparation, together with its use in a sensitive method for the large scale rapid screening of monoclonal antibodies.

The monoclonal antibody technique was invented in 1975, and its preparation process is basically as follows:

(1) Immunizing mice with antigens, (2) Taking spleen lymphocytes and myelomas from the mice and fusing them, (3) Cloning the hybridomas, (4) Screening the specific monoclonal antibodies.

Cloning methods include: (1) The limited dilution method, (2) The soft agar culture method, (3) The single cell microscopic operation method, (4) The monoclonal cell colony microscopic operation method, (5) The fluorescent excitation cell categorizer (flow-type cell meter) separation method.

Classic screening methods for specific monoclonal antibodies include: (1) Enzyme immunoassay, (2) Fluoro-immuno assay, (3) Radio-immunoassay, (4) Chemical light emission immunoassay, (5) Indirect haemagglutination tests. Of these the enzyme immunoassay is used the most, and one type of enzyme immunoassay - Enzyme-Linked Immunosorbent Assay (ELISA) is used here to describe the screening process. An antigen-enveloped carrier is used, and the supernatant fluid from the monoclonal hybridoma culture to be measured is added. If the supernatant fluid contains the corresponding monoclonal antibody, it binds with the enveloping antigen; after washing, a second enzyme-labelled antibody is added to form a second fixed antigen-antibody-enzyme label antibody complex; depending on the strength of the substrate development reaction, qualitative or quantitative analysis can be carried out on the monoclonal antibody specimen to be measured.

The greatest shortcoming of the classic screening methods for specific monoclonal antibodies is that only one antibody from the numerous clones can be screened out, so that there is great waste, and efficiency is low.

By using a protein chip it is possible to detect a large quantity of antigen-antibody binding reactions rapidly. If homogenized tissue is used to immunize mice, and the antibodies secreted by the cloned hybridomas are fixed on a carrier (usually a nylon film, a cellulose nitrate film or glass plate), it is possible to screen for different antigen monoclonal antibody hybridoma strains by using different antigens and their polyclonal antibodies. From a theoretical point of view, if the cloned hybridoma bank is sufficiently large, all the protein monoclonal antibodies can be screened.

The comparative literature and references discovered by searching are appended hereafter.

The objective of this invention is to invent a protein chip, such that the protein chip produced according to its preparation method can be used for the large scale and rapid screening of monoclonal antibodies, the method being simple and the costs being low, together with the simultaneous establishment of a hybridoma bank; this invention also concerns the establishment of a method for using specific antigens and polyclonal antibodies for screening the corresponding monoclonal antibodies.

The following scheme was adopted for the realization of the objective of this invention:

(1) Immunizing BALB/c mice with homogenized tissue;
(2) Fusing spleen cells and myelomas from BALB/c mice;
(3) Separating individual hybridomas with a flow-type cell meter and raising a culture;
(4) Preparing a protein chip used for identifying the antibodies produced while at the same time establishing a hybridoma bank;
(5) Preparing a protein chip for screening use and using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies.

A further concrete description of this invention is given in combination with the appended figures:

The so-called protein chip is an array in which a large quantity of specific antigens (antibodies) are fixed in sequence on a carrier, which can be a cellulose nitrate film, a nylon film or a glass plate, utilizing the antigen-antibody affinity reaction to detect the corresponding antibody (antigen).

The flow chart of this invention and a flow chart of the classic monoclonal antibody preparation are compared in Figure 1, in which 1 is the classic hybridoma antibody preparation flow chart, and 2 is the flow chart for this invention, (1) - (5) in the illustration of this invention being the five operating steps, namely:

(1) Immunizing BALB/c mice with homogenized tissue;
(2) Fusing spleen cells and myelomas from BALB/c mice;
(3) Separating individual hybridomas with a flow-type cell meter and raising a culture;
(4) Preparing a protein chip used for identifying the antibodies produced while at the same time establishing a hybridoma bank;
(5) Preparing a protein chip for screening use and using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies.

In Figure 1:

3. Homogenized tissue 4. Specific antigen 5. Spleen cells 6. Hybridomas 7. Clones 8. Monoclonal antibodies 9. Flow-type separator to separate individual hybridomas 10. Protein chip for identifying the antibodies produced, and establishment of a hybridoma bank 11. Protein chip for screening specific monoclonal antibodies 12. Fusing 13. Myelomas.

The classic preparation method also corresponds to the five steps, as noted above Figure 1.

See Figure 2 for the principle of using the protein chip for identification of the antibodies produced.

In Figure 2:

1. Schemanc diagram of the protein chip for screening specific monoclonal antibodies:

The round black dots indicate the corresponding wells having antibody production; the white round dots indicate no antibody production, as mute clones.

2. On the principle of no development to indicate no antibody production, when there is no mouse antibody IgG (immunoglobulin) in the clones, the anti-mouse IgG antibodies labelled with FITC (fluorescein isothiocyanate) cannot bind and are washed away during the washing, so that the corresponding dots show no fluorescence.

3. On the principle of development to indicate antibody production, when the clones contain mouse IgG antibodies, the IgG will be fixed in specific locations on a glass plate where the hybridoma culture supernatant fluid has been printed as a sequence of dots on the glass plate, with a dot density of 1000 dots/cm^2; FITC-labelled anti-mouse IgG is incubated on the glass plate, and where there are mouse IgG dots they will fluoresce, and by this method a large quantity of mute clones can be conveniently removed.

4 are effective antibodies, 5 are ineffective antibodies, and 6 are FITC-labelled mouse IgG antibodies.

The principle of using the protein chip for the screening of specific antibodies taking AFP (α-foetoprotein) as an example is shown in Figure 3, in which the supernatant fluid from the IgG antibody clones produced is printed in dots on a glass plate with a dot density of 1000 dots/cm^2. AFP and FITC-labelled immune AFP polyclonal antibodies are used, with incubation on the glass plate, so that if certain dots on the glass plates have AFP monoclonal antibodies present, fluorescence will be produced under the excitation light.

In Figure 3, 1 is a schematic diagram of the results of screening for specific monoclonal antibodies with the protein chip, where black indicates positive clones, and white indicates negative; 2 is a diagram showing the principle of no development where there are no antibodies, as non-specific monoclonal antibodies cannot distinguish specific antigens and so cannot form specific monoclonal antibody-antigen-polyclonal antibody-fluorescent complexes, and so the corresponding dots do not fluoresce; 3 is a diagram showing the

principle of development where antibodies are produced, where specific monoclonal antibodies can distinguish specific antigens and so can form specific monoclonal antibody-antigen-polyclonal antibody-fluorescent complexes, so that the corresponding dots show fluorescence; 4 is a specific antibody, 5 is a non-specific antibody, 6 is the antibody for a specific FITC-labelled antigen.

The actual method can be divided into five parts:

1. Immunizing BALB/c mice with homogenized tissue;
2. Fusing spleen cells and myelomas from BALB/c mice;
3. Separating individual hybridomas with a flow-type cell meter and raising a culture;
4. Preparing a protein chip used for identifying the antibodies produced while at the same time establishing a hybridoma bank;
5. Preparing a protein chip for screening use and using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies.

1. Immunizing BALB/c mice with homogenized tissue:

2 g of tissue (all kinds of normal tissue and tumour tissue are acceptable) were taken and formed into a homogeneous paste and centrifuged; the large cell fragments were discarded, and conventional immunization was carried out.

2. Fusing spleen cells and myelomas from BALB/c mice:

The spleen cells and a suitable quantity of myelomas were taken from immune BALB/c mice and fused using PEG (polyethylene glycol); after fusion the cells were immediately transferred to a HAT culture liquid [containing hypoxinthine (H), aminopterin (A) and thymidine (T)], the culture liquid was changed to HT after 4 - 5 days, and after 6 - 7 days it was changed to ordinary culture fluid.

3. Separating individual hybridomas with a flow-type cell meter and raising a culture:

The fused hybridomas were collected and cultured for 7 - 8 days to form a suspension; individual cells were separated using a flow-type cell meter and were collected in a 384 well culture plate. Culture fluid and feed cells (usually mouse stomach macrophages are used) needed first to be placed in the culture wells; they were cultured for 5 days and tested to see whether antibodies had been produced. After culturing for 8 days they were tested once again.

4. Preparing a protein chip used for identifying the antibodies produced while at the same time establishing a hybridoma bank:

(1) Preparation of a protein chip: the supernatant fluid was taken from the cultured cells and transferred to a new 384 well plate. The supernatant fluid from the culture was placed in dots on a glass plate using a specimen dropper to form an array of 1000 dots/cm^2; (different specimen droppers, different dot printing densities and different volumes of supernatant fluid were used); each dot corresponded to a well, and each dot contained 0.2 - 1 μl of supernatant fluid. The IgG concentration was 1 - 100 Pg/ml.

(2) Using the prepared protein chip to identify the antibodies produced while at the same time establishing a hybridoma bank: FITC-labelled anti-mouse IgG antibodies (titre 1 : 50 - 1 : 1000) were added to the protein chip and incubated for 30 - 60 minutes in an incubator at 37°C, and the results were observed under the fluorescence microscope (it is also possible to obtain the image by other means, such as an excitation light scanning instrument).

(3) Establishing a hybridoma bank: dots which fluoresced were selected under the fluorescence microscope; the culture wells containing the corresponding hybridomas were labelled, and a suitable quantity of cells was taken and cultured. The cells were stored in liquid nitrogen to establish the hybridoma bank.

See Figure 4 for the fluorescence photomicrographs showing identification by the protein chip of the antibodies produced. The bright dots correspond to the antibodies produced by the hybridoma, otherwise they are mute clones.

Using FITC-labelled anti-mouse IgG is only one identification method, and other methods include using fluorescent molecule labelling of other anti-mouse IgG polyclonal antibodies or

anti-mouse IgG polyclonal antibodies not labelled and re-coupled with a fluorescent-marked second antibody; another method is firstly to add anti-mouse IgG polyclonal antibodies and couple them to a second antibody labelled with biotin, and then again use an affinity substance labelled with a fluorescent molecule showing fluorescence to raise the sensitivity.

5. Preparing a protein chip for screening use and using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies.

(1) Preparation of a protein chip: a 384 well plate was used with revived frozen cells. They were cultured for 5 - 10 days, and the supernatant fluid was collected and was placed in dots on a glass plate using a specimen dropper to form an array of 1000 dots/cm^2; each dot corresponded to a well, and each dot contained 0.2 - 1 μl of supernatant fluid. The IgG concentration was 1 - 100 Pg/ml, so that many protein chips could be prepared at one time.

(2) Screening for specific monoclonal antibodies:

1) Antigen was added to the protein chip at a concentration of 0.1 - 100 Pg/ml and was incubated for 30 - 60 minutes in an incubator at 37°C;
2) The protein chip was washed 3 - 5 times in a phosphate buffer solution (PBS);
3) Polyclonal antibodies with specific immunity were added to the protein chip, using FITC labelling for the antibodies, giving an antibody titre of 1 : 5 - 1 : 1000, and incubation was carried out for 30 - 60 minutes in an incubator at 37°C;
4) The protein chip was observed under the fluorescence microscope, fluorescing coloured spots were selected, and the corresponding hybridomas were extracted from the hybridoma bank. They were cultured, and the screened monoclonal antibodies were identified;
5) Another antigen and its fluorescent labelled polyclonal antibody were substituted, and steps 1) - 4) were repeated to screen out another monoclonal antibody.

Practical Example:

Homogenized foetal liver was used for immunization to prepare α-foetoprotein (AFP) and actin monoclonal antibodies.

The practical example is divided into five parts:

1. Immunizing BALB/c mice with homogenized foetal liver;
2. Fusing spleen cells and myelomas from BALB/c mice;
3. Separating individual hybridomas with a flow-type cell meter and raising a culture;
4. Preparing a protein chip, discarding mute clones and establishing a hybridoma bank;
5. Preparing a protein chip for screening use, using AFP to screen for AFP monoclonal antibodies and using actin to screen for actin monoclonal antibodies.

1. Immunizing BALB/c mice with homogenized foetal liver:

2 g of foetal liver tissue was taken from an aborted foetus, 10 ml of phosphate buffer solution was added, and the tissue was ground. It underwent ultrasound disruption for 1 - 5 minutes, and was centrifuged to remove the precipitate. It was dialyzed 3 times with 1 L of phosphate buffer solution, and the homogenized paste was concentrated to 1 ml. Mice were immunized with it, being 5 immune BALB/c mice, which were further immunized at intervals of 2 - 4 weeks, and after 4 weeks an immunization booster was given intravenously.

2. Fusing spleen cells and myelomas from BALB/c mice:

(1) Mouse stomach macrophages were prepared as feed cells;
(2) The eyeballs of 5 BALB/c immune mice were removed and blood was taken, and the mice were killed; the spleen was removed under sterile conditions, and a mesh squeezing method was used to obtain dispersed spleen cells;
(3) 36 - 48 hours before fusing, a culture was made of the myelomas;
(4) 1×10^8 spleen cells and $2 - 3 \times 10^7$ myelomas were taken respectively, PEG was added for fusing, and the culture was screened using HAT culture fluid.

3. Separating individual hybridomas with a flow-type cell meter and raising a culture:

(1) A cell suspension was placed in a 70 cm culture dish already supplemented with a feed cell layer, and this was placed in an incubator containing 5 - 8% CO_2 at 37°C for culturing;

(2) The HAT culture liquid from the fusion of that day was cultured and observed once per day. Small colony clones appeared among the fused cells on the fourth day, and HT culture liquid was immediately used instead. After one day it was changed to ordinary complete culture liquid;

(3) Mouse stomach macrophages were prepared as feed cells, and each well of a 384 well plate contained 10^5 feed cells;

(4) On the sixth day after fusion, the hybridomas were very lightly blown and sucked to form a suspension;

(5) Individual cells were separated from the suspension using a flow-type cell meter, and one cell was added to each well, making a total of 100 plates each with 384 wells;

(6) Conventional culturing was carried out for 5 - 8 days, and the growth of the clones was observed. The supernatant fluid was taken from the culture on the eighth day and transferred to a new 384 well plate.

4. Preparing a protein chip, discarding mute clones and establishing a hybridoma bank:

(1) Preparation of protein chip: the supernatant liquid from the culture was dot printed on a glass plate (US Telechem Company, Stock No. SMA-25) using a specimen dropper, with each dot containing 0.2 μl to form a 1000 dots/cm^2 array. Each glass plate was dot printed with 1920 dots (corresponding to 5 plates each with 384 wells). A total of 20 glass plates were dot printed and were dried at room temperature for 24 hours. 100 mg/ml of bovine serum protein was added, and the glass plates were sealed at room temperature for 2 hours and were slowly rinsed for 2 minutes with double distilled water;

(2) Identifying the antibodies produced: 0.3 ml of FITC-labelled anti-mouse IgG was added to the chip with a titre of 1 : 500 and incubated for 45 minutes in an incubator at 37°C. It was then rinsed 3 times in phosphate buffer solution;

(3) Fluorescence microscopy: light excitation at a wavelength of 492 nm was used, and the results were observed and photographed through a 525 nm filter. See Figure 4 for the results, in which the bright spots correspond to the production of hybridoma antibodies, otherwise they are mute clones;

(4) Establishment of hybridoma bank: the cells expressing IgG were selected (a total of 6500 wells), and the cells were suspended and transferred to new 384 well culture plates, totalling 17 plates. They were cultured for 8 days, and the supernatant liquid was transferred to new 384 well plates for preparation of the protein chip. The hybridomas were again cultured, and they were freeze-stored after serial numbering.

5. Preparing a protein chip for screening use, using AFP to screen for AFP monoclonal antibodies, and using actin to screen for actin monoclonal antibodies:

(1) Preparation of protein chip for screening use: the abovementioned culture supernatant liquid obtained was dot printed on glass plates using a specimen dropper, each glass plate having 1920 dots, with a total of 4 glass plates. The dot printing was repeated for 1000 sets, which were dried at room temperature for 24 hours. They were sealed with 100 mg/ml of calf serum for 2 hours and slowly rinsed for 2 minutes with double distilled water, dried and preserved.

(2) Screening for AFP antibodies

1) 0.3 ml of AFP antigen with a concentration of 20 Pg/ml was added to the protein chip and incubated for 45 minutes in an incubator at 37°C. The chip was washed three times with PBS;

2) FITC-labelled anti-AFP polyclonal antibodies (US Chemicon Company, Stock No. AB562) were diluted to a titre of 1 : 100;

3) 0.3 ml of the FITC-labelled anti-AFP polyclonal antibodies was added to the chip and incubated for 45 minutes in an incubator at 37°C;

4) Fluorescence microscopic observation of the chip: the excitation light was at 492 nm, and observation and photography were carried out through a 525 nm filter to record the fluorescent positions under the microscope. See Figure 5 for the results. 2 strains of AFP monoclonal antibodies were screened, indicated by 1 and 2 respectively.

(3) Screening for actin monoclonal antibodies:

The procedure was as in (2), the anti-actin polyclonal antibodies having been purchased from the US Chemicon Company, Stock No. AB978. See Figure 6 for the results. 4 strains of actin monoclonal antibodies were screened, indicated by 1, 2, 3 and 4 respectively. It can be seen from the figure that 1 and 2 showed the highest affinity, and 4 had the lowest affinity.

Advantages of this invention:

Low costs: once the bank is established, the protein chip can be used to screen for the monoclonal antibodies for many antigens, and the overall cost can be reduced by an order of magnitude;

Simple operation: it is not necessary to repeat the immunization of the mice or to repeat the fusion, and many screenings can be carried out after establishment of the bank;

Automation: a flow-type cell meter is used to separate individual hybridomas; manpower requirements are minimised by using the protein chip for identification of antibodies; monoclonal antibodies can be screened rapidly and on a large scale.

Applications of this invention:

Using the protein chip of this invention enables the large scale and rapid screening of monoclonal antibodies, while establishing a hybridoma bank at the same time.

Explanation of Appended Figures to Description:

Figure 1 is a comparison of the flow chart of this invention with the flow chart for the classic preparation of monoclonal antibodies:

1 is a flow chart of the classic preparation of hybridoma antibodies; 2 is a flow chart for this invention. (1) - (5) correspond to the five operating steps.

(1) Immunizing BALB/c mice with homogenized tissue;
(2) Fusing spleen cells and myelomas from BALB/c mice;
(3) Separating individual hybridomas with a flow-type cell meter and raising a culture;
(4) Preparing a protein chip used for identifying the antibodies produced while at the same time establishing a hybridoma bank;
(5) Preparing a protein chip for screening use and using specific antigens and polyclonal antibodies to screen for the corresponding monoclonal antibodies.

In Figure 1:

3. Homogenized tissue 4. Specific antigen 5. Spleen cells 6. Hybridomas 7. Clones 8. Monoclonal antibodies 9. Flow-type separator to separate individual hybridomas 10. Protein chip for identifying the antibodies produced, and establishment of a hybridoma bank 11. Protein chip for screening specific monoclonal antibodies 12. Fusing 13. Myelomas.

The classic preparation method also corresponds to the five steps, as noted in Figure 1 above.

Figure 2 is a diagram of the principle of using the protein chip for identification of the antibodies produced.

1 is a schematic diagram of the protein chip detection results, where black indicates that there is antibody production in the corresponding wells, and white indicates no antibody production, as mute clones.

2 indicates the principle of no development where there is no antibody production. When there is no mouse antibody IgG in the clones, the FITC-labelled anti-mouse IgG antibodies cannot bind with them, and the corresponding dots show no fluorescence.

3 indicates the principle of development where there is antibody production. When the clones contain mouse IgG antibodies, the FITC-labelled anti-mouse IgG antibodies bind with them, and the corresponding dots will fluoresce.

4 are effective antibodies

5 are ineffective antibodies

6 are FITC-labelled anti-mouse IgG antibodies.

Figure 3 is a diagram showing the principle of using the protein chip for the screening of specific monoclonal antibodies.

1 is a schematic diagram of the results of screening for specific monoclonal antibodies with the protein chip, where black indicates positive clones, and white indicates negative clones.

2 is a diagram showing the principle of no development where there are no antibodies, as non-specific monoclonal antibodies cannot distinguish specific antigens and so cannot form specific monoclonal antibody-antigen-polyclonal antibody-fluorescent complexes, and so the corresponding dots do not fluoresce;

3 is a diagram showing the principle of fluorescent development where antibodies are produced, where specific monoclonal antibodies can distinguish specific antigens and so can form specific monoclonal antibody-antigen-polyclonal antibody-fluorescent complexes, so that the corresponding dots show fluorescence.

4 is a specific antibody

5 is a non-specific antibody

6 is the antibody for a specific FITC-labelled antigen

Figure 4 is a fluorescence photomicrograph showing identification by the protein chip of the antibodies produced.

The hybridoma strains corresponding to the bright dots have antibody production, otherwise they are mute clones.

Figure 5 shows the protein chip screening for AFP monoclonal antibodies.

2 strains of AFP monoclonal antibodies were screened, indicated by 1 and 2 respectively.

Figure 6 shows the protein chip screening for actin monoclonal antibodies

4 strains of actin monoclonal antibodies were screened, indicated by 1, 2, 3 and 4 respectively. It can be seen from the figure that 1 and 2 have the highest affinity, while 4 has the lowest affinity.

References:

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